Intraspecies Diversity of the Industrial Yeast Strains *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* Based on Analysis of the Sequences of the Internal Transcribed Spacer (ITS) Regions and the D1/D2 Region of 26S rDNA

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Received November 28, 2006; Accepted March 31, 2007; Online Publication, July 7, 2007

We divided industrial yeast strains of *Saccharomyces cerevisiae* into three groups based on the sequences of their internal transcribed spacer (ITS) regions. One group contained sake yeasts, shochu yeasts, and one bakery yeast, another group contained wine yeasts, and the third group contained beer and whisky yeasts, including seven bakery yeasts. The three groups were distinguished by polymorphisms at two positions, designated positions B and C, corresponding to nucleotide numbers 279 and 301 respectively in the S288C strain. The yeasts in the Japanese group had one thymine at position B and one thymine at position C. The wine yeasts had one thymine at position B and one cytosine at position C. And the beer and whisky yeasts had two thymines at position B and one cytosine at position C. Strains of *S. pastorianus* were divided into three groups based on the sequences of their 26S rDNA D1/D2 and ITS regions.

Key words: industrial yeast; 26S rDNA D1/D2; internal transcribed spacer regions; intraspecies diversity

Traditionally, the classification and identification of yeast species and strains have been based on characteristics such as morphological traits and on physiological abilities. The process of characterization is laborious and time consuming. Molecular techniques utilizing amplification of target DNAs provide alternative methods for identification. Ribosomal RNAs (rRNA) provide a powerful taxonomic indicator, because they are highly conserved and are universally found in living cells. The genes coding for yeast rRNA occur as tandem repeated units on chromosome XII, with 18S, 5.8S, and 26S rRNA genes co-transcribed. Sequence comparisons of the genic regions in the rDNA repeat unit of various organisms have shown a relatively high degree of evolutionary conservation, and have been used as bases for inferring phylogenetic relationships. Phylogenetic relationships among the ascomycetous yeasts were analyzed from the 5' end of the 26S rDNA D1/D2 region sequence divergence. Between the coding regions are the internal transcribed spacer 1 and 2 regions (ITS1 and ITS2 respectively) which evolve more rapidly and therefore vary among different species within a genus. The same yeast species were identified by restriction fragment length polymorphism (RFLP) using the polymerase chain reaction (PCR) technique of ITS regions. The PCR products of ITS regions digested with restriction endonucleases yielded a specific restriction pattern for each species. Industrial yeast strains of *Saccharomyces cerevisiae* are essential in the production of fermented products such as sake, shochu, wine, beer, whisky, and bakery items. Industrial yeast strains of *Saccharomyces pastorianus* are used for lager fermentation. *S. pastorianus* is thought to have originated from a natural hybridization event that occurred between an *S. cerevisiae* strain and a non-*S. cerevisiae* strain, probably an *S. bayanus* strain. These industrial yeasts contain both common and distinct characteristics; those for brewing and distilling are similar, but are different from those for wine making or baking. In an analysis of the amplified fragment length polymorphism (AFLP) of industrial yeast strains, *S. cerevisiae* strains separated into three subclusters: one consisting of sake, shochu strains, and one of a whisky strain, one consisting of bakery, wine, beer, and two whisky strains, and one consisting of...
laboratory strains. To date, industrial yeasts have not been divided according to sequences in the 26S rDNA D1/D2 region and the ITS regions. In this study, we investigated sequences of 26S rDNA D1/D2 and the ITS regions and divided industrial yeasts into separate classes.

Materials and Methods

Strains and amplification of target sequences. The yeasts used in this study (Table 1 and Table 2) were grown overnight on YPD (2% glucose, 1% yeast extract, 2% peptone) at 30 °C. Genomic DNA was isolated using Dr. GenTLE for Yeast (Takara, Kyoto, Japan), then dissolved in sterile water. The primers used for amplification of the 26S rDNA D1/D2 region were NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′), and the ITS regions, ITS5 (5′-GGAAGTAAAAGTCGTAACAA-3′) and ITS4 (5′-TCTTCCGCTTATTGATATGC-3′).5,12) The amplification reaction was performed in a 100 μl solution containing template DNA and forward and reverse primers using TaqEX (Takara), according to the manual.

Sequence analysis. Amplified DNA fragments of the 26S rDNA D1/D2 and ITS regions were purified using a PCR purification kit (Qiagen, Hilden, Germany). The sequences of PCR products were determined using the BigDye Terminator Cycle Sequencing V1.1 Kit and the ABI 310 automatic DNA sequencer directly (Applied Biosystems, Foster City, CA). The primers used for sequencing were NL1, NL4, ITS5, ITS4, ITS3 (5′-GCATCGATGAAGAACGCAGC-3′), and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′).12) For S. cerevisiae strainsNCYC 1245, KJ, and SA, and S. pastorianus strains Weihenstephan 34/70 and IFO 1962, amplified PCR products were cloned into pGEM-T vector. The nucleotide sequence data reported are available in the DDBJ databases (http://www.ddbj.nig.ac.jp/Welcome-j.html) under the accession numbers indicated in Tables 1 and 2. The sequences of the ITS regions of S. cerevisiae strains IFO 304, IFO 2112, and IFO 2114 and S. pastorianus strains IFO 613T and IFO 1167 were
obtained from DDBJ, and the sequence of S288C was obtained from the Saccharomyces Genome Database (SGD) (http://www.yeastgenome.org/).

Results

Sequence of 26S rDNA D1/D2 region

All sequences of the 26S rDNA D1/D2 region of industrial yeast strains of *S. cerevisiae* were identical. These sequences were all the same as the corresponding sequence of *S. cerevisiae* S288C in the database.

Three types of 26S rDNA D1/D2 sequence were found in *S. pastorianus* strains. One type, found in Weihenstephan 34/70 and IFO 10011, was the same as the *S. cerevisiae* sequence. Another type, found in IFO 613 and IFO 1167, was the same as the sequence of *S. bayanus* type strain CBS 380 in the DDBJ database (accession no. AF113892). The third type, found in IFO 1962, had sequences of both *S. cerevisiae* and *S. bayanus*, as above.

Sequence of ITS regions and diversity of industrial yeasts

Four nucleotide positions in ITS1 (positions A, B, C, and D) and two nucleotide positions in ITS2 (positions E and F) were polymorphic among the 30 strains of *S. cerevisiae* and five strains of *S. pastorianus* examined in this study (Fig. 1, Table 1). At positions A, B, E, and F, the number of thymines was different among the strains. At positions C and D, some strains had thymine and the others had cytosine.

Industrial strains of *S. cerevisiae* were divided into three groups based on the sequences of their ITS regions. The sequences of the ITS regions of sake yeasts and shochu yeasts were the same. Because all of the sake yeasts and shochu yeasts and one kind of bakery yeast (SK) were isolated in Japan, we designated them the Japanese group of yeasts.

We did not determine the complete ITS1 and ITS2 sequences of 11 strains (NCYC 1245, NCYC 1333, NCYC 2347, JP, KR, KK, KJ, and SA) using the direct sequence method because of the variable number of thymines in each strain at positions A, B, and E. Hence, the amplified PCR products of the ITS regions of some of the strains were cloned into pGEM-T vector, and then the sequences were analyzed (Table 3). Eleven clones of NCYC 1245 were sequenced. Eight clones had 12 thymines, two clones had 11 thymines, and one clone had 10 thymines at position A. The sequence of 12 thymines was the major sequence determined by the direct sequence method. Hence, the major sequence determined by the direct sequence method is indicated in Table 1, and was registered in the database for strains NCYC 1333, NCYC 2347, JP, KR, KK, KJ, and SA.

Three clones of the ITS regions of strains KJ and SA were sequenced (Table 3). Although all clones had 13 thymines at position A, SA had a variable number of thymines according to the results of the direct sequence method (Table 1). KJ had either one thymine or two at position B. Although all clones had two thymines at position B, SA had either one thymine or two according to the results of the direct sequence method.

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**Table 2. S. pastorianus Strains Used in This Study and Differences in ITS Regions**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Type</th>
<th>ITS1 Number of nucleotides at each position*</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weihenstephan</td>
<td>Beer (Lager)</td>
<td>14T** 2T 1C 1T 10T** 6T</td>
<td>AB279758</td>
</tr>
<tr>
<td>34/70</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFO 10011</td>
<td>Beer (Lager)</td>
<td>14T** 2T 1C 1T 10T** 6T</td>
<td>AB279757</td>
</tr>
<tr>
<td>IFO 1962</td>
<td>Beer (Lager)</td>
<td>12T** 2T** 1C, 1T 1T 10T** 6T</td>
<td></td>
</tr>
<tr>
<td>IFO 613Txxxx</td>
<td>Beer</td>
<td>7T 1T 1T 1T 8T 6T</td>
<td>D89888</td>
</tr>
<tr>
<td>IFO 1167xxxxxx</td>
<td>Brewery</td>
<td>7T 1T 1T 1T 8T 6T</td>
<td>D89889</td>
</tr>
</tbody>
</table>

*Numbers of thymines (T) and cytosines (C) are indicated.

**Values show the numbers of thymines as reported in the database; however, in the sequences determined in this study, the number of thymines was variable.

***Type strain of *S. pastorianus*.

****Type strain of *S. carlsbergensis*.**
At position A, the yeasts in the Japanese group and the wine yeasts had seven thymines (Table 1). The other strains had more thymines. Moreover, eight strains (NCYC 1245, NCYC 1333, NCYC 2347, JP, KR, KK, KJ, and SA) had different numbers of thymines at position A. At position B, the yeasts in the Japanese group and the wine yeasts had one thymine (Table 1). The other strains had two thymines. At position D, three of the bakery yeasts (KY, NN, and JP) and whisky yeast IFO 2106 had one cytosine. At position E, the yeasts in the Japanese group and the beer yeasts (NCYC 1245 and NCYC 1333) had nine thymines (Table 1). The wine yeasts had eight thymines. The other strains had 10 thymines. Moreover, three bakery strains (KK, KJ, and SA) had sequences of different numbers of thymines at position E. At position F, all of the wine yeasts except for B4 had seven thymines, two of the beer yeasts (NCYC1245 and NCYC1333) had eight thymines, and the other strains had six thymines (Table 1).

The \textit{S. pastorianus} strains fell into the same three groups, characterized by sequences of the 26S rDNA D1/D2 region (Table 2). One was the \textit{S. cerevisiae} type (Weihenstephan 34/70 and IFO 10011). These strains, like the beer and whisky strains, had two thymines at position B and one cytosine at position C. They had the same ITS sequence as \textit{S. cerevisiae} NCYC2347, except for position E. In view of the variable number of thymines at positions A and E, the amplified PCR products of the ITS regions of Weihenstephan 34/70 were cloned, and then the sequences were analyzed (Table 3). The sequences of 11 clones were determined; one clone had 15 thymines, five clones had 14 thymines, four clones had 13 thymines, and one clone had 12 thymines at position A. Eight clones had 10 thymines, two clones had nine thymines, and one clone had eight thymines at position E. Another type was a non-\textit{S. cerevisiae} type that had the same sequence as \textit{S. bayanus} (IFO 6137 and IFO 1167). The third type had both sequences for positions B and C, and the number of thymines was variable at positions A and E (IFO 1962). The PCR products of the ITS regions of strain IFO 1962 were cloned, and then the sequences were analyzed (Table 3). The sequences of six clones were determined, and six clones had 12 thymines at position A. Five clones had two thymines and one clone had one thymine at position B. Three clones had 10 thymines, one clone had nine thymines, and two clones had eight thymines at position E. Although all the clones had 12 thymines at position A, IFO 1962 had a variable number of thymines according to the results of the direct sequencing method (Table 2).

Strains B4, K1(V1116), and Weihenstephan 68 differed slightly from the sequence of S288C in addition to the polymorphisms at positions A–F. B4 and Weihenstephan 68 had one nucleotide difference, and K1(V1116) had one additional adenine. These differences were excluded from the criterion of diversity in this study.

### Discussion

In this study, we found that industrial yeasts \textit{S. cerevisiae} can be divided into three groups according to the sequences of the ITS regions. One group included Japanese yeasts such as sake yeasts, shochu yeasts, and one kind of bakery yeast, which was isolated from a natural source in Japan. Another group included wine yeasts. The third group included beer and whisky yeasts, including seven kinds of bakery yeast. The three groups can be distinguished by two polymorphisms, designated position B and position C. Yeasts in the Japanese group had one thymine at position B and one thymine at position C. Wine yeasts had one thymine at position B and one cytosine at position C. Beer and whisky yeasts had two thymines at position B and one cytosine at position C.

The sequences of the ITS regions of yeasts in the Japanese group, such as sake, shochu, and one kind of bakery yeast, were the same. We also investigated the sequence of sake yeasts K1, K3, K4, and K5, which are not commercially available now, whose sequences were the same as other sake yeasts (data not shown). The bakery yeast SK was isolated from a natural source in Japan, which had a low potential for maltose fermentation as compared to other commercially available bakery yeasts. In another study, we isolated several \textit{S. cerevisiae} strains from natural sources in Japan because the sequence of the 26S rDNA D1/D2 region was identical to the sequence of \textit{S. cerevisiae} (data not shown). These yeasts also had a low potential for maltose fermentation. These ITS sequences were identical to the ITS sequences of yeasts in the Japanese group (data not shown). Therefore, the fact that these yeasts came from the same geographical location (Japan) might explain why they have the same ITS sequences.

The wine yeasts differed from the other groups in having one thymine at position B, one cytosine at

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**Table 3. Numbers of Clones of the Sequence-Bearing Variable Number of Thymines**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number of clones sequenced</th>
<th>Position</th>
<th>Number of thymines (number of clones)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCYC 1245</td>
<td>11</td>
<td>A</td>
<td>12 (8); 11 (2); 10 (1)</td>
</tr>
<tr>
<td>KJ</td>
<td>3</td>
<td>A</td>
<td>13 (1); 12 (1); 11 (1)</td>
</tr>
<tr>
<td>SA</td>
<td>3</td>
<td>A</td>
<td>13 (3)</td>
</tr>
<tr>
<td>B</td>
<td>2 (3); 1 (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weihenstephan 34/70</td>
<td>11</td>
<td>A</td>
<td>15 (1); 14 (5); 13 (4); 12 (1)</td>
</tr>
<tr>
<td>IFO 1962</td>
<td>6</td>
<td>E</td>
<td>10 (8); 9 (2); 8 (1)</td>
</tr>
<tr>
<td>B</td>
<td>2 (5); 1 (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>10 (3); 9 (1); 8 (2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
position C, and eight thymines at position E. B4 was different in that it had six thymines at position F and a nucleotide difference at a position in the ITS1 region. The laboratory strain S288C had the same ITS sequence as strains B1 and OC-2.

Based on ITS sequences, bakery yeasts were in the same group as beer yeasts. Since the early records of the history of fermentation show that foam from beer fermentation was used to raise dough, bakery and beer yeasts may have originated from the same ancestral strains. A high potential for maltose fermentation is necessary for both brewing and fast dough fermentation. This suggests that beer, whisky, and bakery yeasts are in the same group. However, another study using principal-component analysis showed that beer yeasts were clearly differentiated from bakery yeasts. The authors of that study stated that beer yeasts are highly adapted to maltose fermentation but possess poor leavening ability. Beer and bakery yeasts appear to have changed their characteristics to adapt to the environment even though they originated from the same ancestral strain. Moreover, commercially available bakery yeasts have a variable number of thymines at positions A and E. This might be because bakery yeasts are homothallic, with a high, irregular degree of polyploidy.

In a study of analysis of the amplified fragment length polymorphism (AFLP) of industrial yeast strains, S. cerevisiae strains separated into three subclusters. The results of that study indicated that the sake and shochu strains are closely related, in agreement with our finding that these strains are in the same group. Although the AFLP results indicated that the wine, beer, bakery, and whisky strains are closely related, our study using ITS sequences can separate wine strains from beer, bakery, and whisky. Moreover, Azumi et al. found that laboratory strains, including S288C, shared relatively few fragments with the industrial strains. However, strain S288C had the same ITS sequence as strains B1 and OC-2 in our study.

S. pastorianus strains were divided into three groups according to their 26S rDNA D1/D2 and ITS sequences. One is the S. cerevisiae type. These strains had the same ITS sequence as S. cerevisiae except for the polymorphisms at positions A–F. Another is a non-S. cerevisiae type that had the same sequence as S. bayanus. The third type (IFO 1962) had both sequences for positions B and C, and the number of thymines was variable at positions A and E. This result is consistent with the hypothesis that S. pastorianus originated from a natural hybridization event that occurred between a S. cerevisiae strain and a non-S. cerevisiae strain.

Acknowledgments

The authors thank Nami Goto-Yamamoto and Nobuhiko Mukai for providing some of the strains used in this study.

References